Towards personalised treatment of patients with colorectal liver metastases

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Chapter 8

Summary, discussion and future perspectives

Part I: Association of biomarkers in resected colorectal liver metastases with patient survival

The first aim of this thesis was to identify biomarkers able to help predict patient survival after surgery for colorectal liver metastases (CRLM). This is relevant because accurate prognostication can guide management for the individual patient, such as the administration of adjuvant chemotherapy after liver surgery and a patient-tailored follow-up (intensity, duration). As high-throughput techniques allow for much wider screening for potential biomarkers compared to previous studies, it is important to review the results of these high-throughput studies. In addition, although there is growing evidence for the importance of immunological processes in cancer behaviour, there have been no recent reviews on the topic of CRLM immunology. Therefore, in **chapter 2**, we reviewed the literature on prognostic immunological and molecular markers studied in tumour tissue obtained from surgical resection for CRLM. This analysis did not yield a biomarker that would be able to predict survival with high accuracy which technically and logistically could be easily applied in patient care. However, the quantification of intra-tumoural CD3+ T cells and tumoural CXCR4 expression showed the most significant associations with patient survival. High infiltration of CD3+ T-cells was associated with a favourable survival and high CXCR4 expression with an unfavourable survival. Nevertheless, it was not possible to accurately predict patient survival based on tissue-based immunophenotypical markers.

In **chapters 3** through **5**, we aimed to identify prognostic biomarkers by analysing mRNA expression, protein expression, microRNA expression and DNA copy number variation. We aimed to improve previous study designs by using more advanced experimental methods and stricter patient inclusion criteria. We compared two patient groups: poor survivors (who died of recurrences within 30 months after surgery) vs. good survivors (those alive without recurrences more than 60 months after surgery).

In **chapter 3**, we show that high expression of immune-related and stroma-related markers in tumour samples was associated with good patient survival. Previously, four independent mRNA signatures had been published that aimed to predict patient survival after surgery for CRLM [1–4]. However, no single gene was shared between the four published signatures, and our own mRNA expression study also showed no overlap with the other signatures (**chapters 2 and 3**). This lack of shared genes might be explained by inter-study differences, as these studies used different experimental methods and had different patient inclusion criteria and treatment strategies. As discussed in **chapter 3**, previous studies used microarrays while we used mRNA sequencing [1–4]. In addition, the rate of administrating chemotherapy differed widely between studies [1,2,4], and this

might cause bias because chemotherapy can influence tumour biology and the natural course of the disease [5]. To avoid this particular type of (potential) bias, we did not include patients treated with neoadjuvant or adjuvant chemotherapy in any of our studies (**chapters 3-5**).

In **chapter 3,** we demonstrated using immunohistochemistry that high stromal infiltration of CD79A+ B cells and Kappa/Lambda+ plasma cells might be associated with good survival. This finding questions the role of anti-tumour B-cell immunity. As yet there is no consensus: while some authors state that immunoglobulin-production by plasma cells and antigen presenting B-cells can enhance anti-tumour immunity, others state that B-cells are simply bystanders that are not functional [6,7]. In contrast, B-cell-mediated immune suppression, which promotes cancer growth, has also been reported [8]. Additionally, a desmoplastic growth pattern, which is characterised by a rim of stromal cells between the tumour and the liver parenchyma, might be associated with a good patient survival. Future evaluation on larger cohorts is necessary to firmly establish whether these prognostic biomarkers can add value to the current clinical models.

In **chapter 4**, we analysed microRNA expression levels in our cohort and found that high expression of miR-196b and miR-19b was associated with poor survival. The number of studies on the prognostic role of microRNAs after surgery for CRLM rapidly increased in recent years (**chapters 2 and 4**). All the previous studies used high-throughput methods to find prognostic markers, and similar to the mRNA studies, no overlap in prognostic miRNAs was observed. Our study showed overlap with one of these studies, but with an effect in the opposite direction. We observed an association between poor patient survival and high miRNA 196b-5p expression, while Li et al. [9] had observed poor patient survival in tumours with low miRNA 196b-5p expression This disagreement between studies might be caused by differences in patient inclusion criteria, as Li et al. did not report on neoadjuvant chemotherapy or show a multivariable analysis to correct for clinicopathological factors [10].

In **chapter 5**, we studied copy number variation and showed that a loss of chromosome 22 was independently associated with a poor patient survival. Although a loss of chr22 is not an uncommon feature in oncology, the biological consequence of this phenomenon is not well-understood [11,12]. We tried to address a potential biological downstream effect of a loss of chr22 by analysing the relation with our mRNA expression data (**chapter 3**). Using pathway analysis, we showed that genes related to the immune system are associated with a loss of chr22. These findings are comparable to the findings in **chapter 3**, which strengthens the hypothesis on the association between patient survival and the immune system. Whether there is a causal relation between loss of chr22 and a lower immune response is still in question.

Importance of multivariable analysis

In **chapters 3** through **5**, we performed multivariable analyses to correct for known clinical and molecular risk factors to assess the prognostic value of the studied biomarkers. The clinical risk score (CRS)[13], which includes five tumour characteristics, is known to be associated with patient survival and development of recurrences in large cohorts. In addition to these clinical risk factors, we also analysed known molecular risk factors in our patient cohorts, namely microsatellite instability status and KRAS/BRAF mutations. Previous research has shown that patients with tumours that harbour a *BRAF* V600E mutation have an unfavourable survival compared to BRAF wildtype tumours [14]. Similarly, harbouring a *KRAS* (codon 12 and 13) mutation is associated with an unfavourable survival and a lack of response to anti-EGFR treatment [15–20]. However, these molecular risk factors were not associated with patient survival in our analyses and did not therefore bias the biomarkers we studied (**chapters 3-5**). One might argue that our cohorts are too small to detect a survival advantage for these known molecular markers. However, as we aimed to predict survival for the individual patient, we worked to find biomarkers with larger effect sizes than currently known biomarkers. In multivariable analyses, we found that low stromal infiltration of CD79A+ B cells (**chapter 3**), low stromal infiltration of K/L+ plasma cells (**chapter 3**), a non-desmoplastic growth pattern (**chapter 3**), high miR-19b expression (**chapter 4**) and a loss of chr22 (**chapter 5**) might be predictors of poor patient survival. In addition, clinicopathological factors like a high CRS [13] (**chapter 4**) and male sex (**chapters 3 and 4**) were also associated with poor patient survival. It has been reported that males had a poorer survival [21,22] and more often had early recurrences [23] after surgery for CRLM compared to females.

Our total cohort of patients treated in the University Medical Centre Groningen (UMCG) consisted of 48 patients, but we were not able to include all 48 tumour samples in all experiments. By combining the data from **chapters 3-5**, we are able to show the prognostic value of all studied biomarkers in a subset of 32 patients. For this set of patients, we carried out a multivariable analysis including the biomarkers studied in **chapter 3** (IHC), **chapter 4** (microRNA) and **chapter 5** (CNV). Table 1 shows that six factors had a p-value <0.1 in univariable analysis, and these factors were carried over to the multivariable analysis. As miR-19b and miR-17 expression were correlated ($r=0.678$, $p<0.001$), we only included miR-19b in the multivariable analysis. The multivariable model - which included the size of the largest CRLM, desmoplastic growth pattern, miR-19b expression, a loss of chr10 and a loss of chr22 - had a Nagelkerke R square of 0.637 and predicted 27 out of 32 patients (84.4%) in the correct survival group (table 1). In comparison, the five factors in the CRS together had a Nagelkerke R square of 0.157 and predicted only 17 out of 30

patients (56.7%, 2 missing CEA values) in the correct survival group. The only independent prognostic factor in this cohort of 32 patients was a loss of chr22 (p = 0.028, table 1), which on its own predicted more patients in the correct survival group than the five CRS factors together (23/32 vs. 17/32).

We focussed on tissue-based molecular markers that could add prognostic value to the current clinical risk models. But what do we accept as accurate prognostication? As biology does not play by statistical rules, prognostic models will probably never reach 100% accuracy. In statistics, we generally accept a 5% chance that our observation is different from the total population. Our multivariable model of 32 patients including only 5 factors (table 1) placed 84.4% of the patients in the correct survival group, which means the model needs to improve by approximately 10% to reach 95% accuracy. Although we and others have analysed multiple biological entities to improve prognostication, some topics remain unexamined, e.g. genome-wide DNA mutation analysis and long non-coding RNA expression. Great promise lies in a multi-omics approach using large cohorts that integrates several types of molecular data. The (future) models with the highest prognostic value are likely to include genomic, epigenomic, transcriptomic and proteomic data [24,25]. In addition, as shown in this thesis, clinicopathological characteristics are a very important part of prognostication as well. Molecular and clinical data together might be able to predict survival rates and the development of recurrences by exceeding 95% accuracy, which would make them of value for the individual patient.

Table 1 Multivariable analysis of the 32 patients in whom all analyses were performed

	Univariable	Multivariable	
	P value	OR (95% CI)	P value
Patient characteristics			
Mean age at liver surgery	0.286		
Male sex	0.137		
Tumour characteristics			
Size largest CRLM	0.074	0.751 (0.543-1.038)	0.083
Rectal primary tumour	0.174		
Major liver surgery	0.116		
Clinical risk score			
Interval CRLM < 12 months	0.465		
CEA >200 mg/µl	0.417		
More than 1 CRLM	0.313		
CRLM larger than 5cm	0.530		
N+ primary tumour	0.137		

Table 1 Multivariable analysis of the 32 patients in whom all analyses were performed (continued)

	Univariable	Multivariable	
	P value	OR (95% CI)	P value
Molecular tumour characteristics			
Microsatellite instable	0.927		
KRAS (codon 12/13) mutation	0.648		
BRAF V600E mutation	1.000		
Immunophenotypical markers			
General lymphocytes			
High CD45 tumour stroma	0.893		
High CD45 invasive margin	0.514		
T-cells			
High CD4 tumour stroma	0.466		
High CD4 invasive margin	0.946		
High CD8 tumour stroma	0.982		
High CD8 invasive margin	0.320		
High CD8 intra-tumoural	0.648		
High FOXP3 tumour stroma	0.172		
High FOXP3 invasive margin	0.999		
B-cells			
High CD79A tumour stroma	0.167		
High CD79A invasive margin	0.391		
High K/L tumour stroma	0.137		
High K/L invasive margin	0.268		
High SLAMF7 tumour stroma	0.540		
High SLAMF7 invasive margin	0.723		
100% desmoplastic growth pattern	0.046	0.059 (0.003-1.094)	0.058
MicroRNA qPCR			
High miR-196b expression (> median)	0.154		
High miR-19b expression (> median)	0.082	9.099 (0.710-116.669)	0.090
High miR-17 expression (> median)	0.051		
Copy number variation			
Loss chr10	0.086	3.576 (0.392-32.597)	0.258
Loss chr22	0.018	18.026 (1.366-237.927)	0.028
Percentage of genome changed	0.122		

Odds ratio >1 corresponds to poor patient survival. OR = Odds ratio, CI = confidence interval, CRS = clinical risk score, CRLM = colorectal liver metastases, CEA = carcinoembryonic antigen, N+ = lymph node positive, K/L = Kappa/Lambda.

Novelty

We identified novel biomarkers that were independently associated with patient survival. A high miR-19b expression (**chapter 4**) and loss of chr22 (**chapter 5**) were predictive of poor survival. Eight other studies analysed prognostication by microRNA expression, and none reported a prognostic value for miR-19b expression [10,26–32]. Similarly, we are the first group to report an association between loss of chr22 and patient survival [33–35]. Unfortunately, although many studies present prognostic molecular markers, only a few present the same marker as being prognostic (**chapters 2-5**). This is probably partly the result of inter-study differences, e.g. differences in inclusion of patients, treatment regimens and experimental methods. Novel prognostic biomarkers therefore need to be validated in other cohorts before they can be introduced in clinical care. To improve and accelerate this process, collaborations between research groups should be the rule rather than the exception. For example, uniform inclusion criteria for patients should be agreed upon to establish repeatable results in a second cohort. Alternatively, despite proper statistical analyses, previously identified markers (or ours!) may be false positive findings and are therefore hard to replicate. Still, it is interesting that multiple studies show prognostic value for immune-related markers (**chapters 2 and 3**).

Future perspectives

We aimed to predict patient survival after surgery for CRLM by measuring the underlying tumour biology. The molecular markers we studied might improve prognostication when our findings are replicated in other cohorts. Our findings also raise questions about the biological function of these markers that have yet to be answered. For example, what is the specific function of B cells in tumour immunology (**chapter 3**)? Which cells are responsible for creating a desmoplastic rim around the tumour (**chapter 3**)? What are the tissue-specific mRNA targets of miR-19b and miR-196b and how do they influence tumour biology (**chapter 4**)? Does the association between a loss of chr22 and immune-related mRNA expression also imply causality (**chapter 5**)? Future studies may strive to elucidate these research questions. However, this does not impair the application of these biomarkers in clinical care. These prognostic biomarkers might be incorporated in future guidelines if extensive replication in other cohorts shows accurate prognostication.

Accurate prognostication after surgery for CRLM might guide further management for the individual patient, e.g. guiding the administration of adjuvant chemotherapy after surgery and a patient-tailored follow-up. While adjuvant chemotherapy after liver surgery is often a standard treatment in hospitals in the USA, this is not the case in the Netherlands. A randomised controlled trial showed no benefit in overall survival for patients receiving

perioperative FOLFOX4 + liver surgery vs. liver surgery alone [36]. In contrast, the study did show a marginal benefit in progression-free survival (PFS) for patients receiving perioperative chemotherapy, suggesting that a subset of patients might benefit from chemotherapy. The question remains whether this marginal PFS benefit can outweigh the associated morbidity and costs for large cohorts. Therefore we propose that a more personalised approach should be developed in which prognostication might guide adjuvant treatment. For example, patients who are highly likely (>95%) to develop a recurrence can be administered adjuvant chemotherapy, while patients who are highly likely (>95%) to be cured by liver surgery can be spared the chemotherapy.

Part II: The role of thermal ablation in the management of patients with colorectal liver metastases

In 75% of all CRLM patients, curation is not possible because of widespread liver involvement, extra-hepatic disease or comorbidity [37]. For these patients, only palliative chemotherapy or best supportive care can be offered. Radiofrequency ablation (RFA) extends the criteria for intentionally curative treatment and therefore increases survival rates in the entire population of patients with CRLM [38].

The second aim of this thesis was to study the role of targeted treatment by thermal ablation in patients with CRLM, focussing on patient survival. In **chapter 6**, we concluded that RFA, in particular via the percutaneous route, increased the number of patients who can be retreated for recurrent CRLM. A repeat intervention was more often possible due to treatment with RFA, which can be applied multiple times in a series. Likewise, percutaneous RFA was progressively more frequently applied with each additional intervention for recurrence of liver metastases. Multivariable analysis showed that, in this large cohort, the known clinical factors included in the CRS were independently associated with overall survival. In **chapter 7**, we analysed the impact of RFA in simultaneous surgery for both the primary colorectal cancer (CRC) and synchronous CRLM in one surgical session. We found that RFA can be applied safely and successfully in adjunct to resection of the primary CRC and CRLM. Patients who underwent RFA + partial liver resection had a lower complication severity compared to patients who only underwent a partial liver resection. In addition, the type of surgery for the primary tumour turned out to be critically associated with morbidity. Patients who underwent an abdominoperineal resection more often suffered from postoperative complications. Although patients more often developed recurrences after treatment with RFA (**chapter 6**), we did not observe differences in survival rates

comparing RFA with partial liver resection (**chapters 6 and 7**). Therefore we concluded that RFA is a very useful addition to the treatment armamentarium in the surgical management of CRLM.

Patient selection

There is considerable variation in survival rates among studies that compare RFA with partial liver resection, which remains the gold standard treatment for CRLM. In most studies, patients undergoing a partial liver resection had more favourable survival rates compared to RFA [39–44]. A recent meta-analysis compared RFA + partial liver resection vs. partial liver resection alone in 1,918 patients and concluded that there was no significant survival advantage for either treatment (HR = 1.24, 95%CI = 0.84-1.84). The application of RFA increased the number of patients who were treated with curative intent and did not compromise survival rates. They also compared RFA vs. partial liver resection in 1,824 patients and showed a survival advantage for patients undergoing a partial liver resection (HR = 1.78, 95%CI = 1.35-2.33). Unfortunately, they did not take clinicopathological characteristics into account, as the authors of the meta-analysis did not have access to this patient data. In our cohort, patients undergoing a partial liver resection + RFA had unfavourable clinicopathological characteristics (**chapters 6 and 7**) associated with unfavourable survival rates (data not shown). The selection of suitable patients for treatment might therefore already explain the differences in survival rates, independent of the treatment chosen.

Biological effect of thermal ablation

Although thermal ablation is included in the Dutch national guidelines as an intentionally curative treatment of CRLM [45], the molecular effect of eradicating the tumour by periods of heat is not completely understood. The seemingly obvious effect of thermal ablation is the physical destruction of tumour cells by hyperthermic injury. However, this process is not as obvious as it may seem. The central zone around the tip of the ablation needle reaches temperatures above 50° Celsius and quickly undergoes coagulative necrosis. The transitional zone reaches temperatures between 41-45° Celsius, which leads to reversible damage and cellular apoptosis [46,47]. In response, increased blood flow and chemokines are produced, attracting immune cells to the injured site [47]. Tumour antigens that are released after tumoural necrosis are taken up by antigen presenting cells, in particular dendritic cells, to enhance the immune response [48]. One of the catalysts in the antigen presenting process is heat shock protein 70 (HSP70), which can also serve as a cytokine that stimulates macrophages and dendritic cells to produce more cytokines [49]. As discussed

in **chapter 6**, patients who underwent RFA more often developed recurrences compared to patients who underwent surgery. This might be due to incomplete eradication of all tumour cells, but one could also speculate that the effect of thermal ablation can be pro-tumourigenic. Rozenblum et al. showed in two mouse studies that tumour load is higher after thermal ablation [50,51]. Their first study described tumour-bearing livers in which normal liver parenchyma was ablated, resulting in larger tumours one month after ablation compared to sham-operated mice [50]. The second study showed that the proliferative markers CDC47 and BrdU were expressed more highly in healthy liver parenchyma after RFA, both in the ablated and in the untreated liver lobe [51]. Thus, the accelerated growth of tumour cells after RFA might be a hypoxia-driven phenomenon [52]. Future research is warranted to study the balance between the anti-tumourigenic immune response and the pro-tumourigenic proliferation after thermal ablation. The role of the immune system in thermal injury might be used to improve systemic anti-tumour immunity, e.g. by combining ablation with immunotherapy [46,47].

Future perspectives

With the good results of observational studies, especially our study in which patients with recurrences after previous liver resection can obtain significant survival, it is questionable whether a randomisation between resection and ablation is ethical. Anyway, currently two randomised trials are running at this moment comparing thermal ablation vs. partial liver resection for small resectable CRLM. The COLLISION trial aims to study thermal ablation in comparison to partial liver resection in patients with at least one resectable and ablatable CRLM and no extrahepatic disease [53]. The primary endpoint is overall survival. The aim is to include 687 patients over a timespan of three years at multiple hospitals in the Netherlands, and will present their final results after 10 years of follow-up, which will be around the year 2031 [53]. In addition, the LAVA trial has a similar aim to that of the COLLISION trial, but specifically studies patients who are at high surgical risk because of their age, comorbidity or tumour load. LAVA aims to include 330 patients and will perform follow-up for five years [54]. Of note, there is considerable variation in the amount of experience individual centres have with thermal ablation, which might have negative influence on the outcome of the patients that underwent ablation. The MAVERRIC trial is a prospective multi-institutional study that aims to analyse the strategy of first line computer-navigated percutaneous microwave ablation (MWA) vs. partial liver resection in tumours ≤ 3cm. This multicenter trial started in 2015 and recruitment is performed at the Karolinska Institutet, Sweden, at the University Hospital Bern, Switzerland, and at the University Medical Center Groningen, the Netherlands. These centers have extensive

experience with ablation of liver tumors and especially with the application of navigation guided ablation. The aim is to include a total of 100 participants and compare the 3-year survival rate with matched patients who underwent open or laparoscopic resection of liver metastases identified in the Swedish liver registry. The results of the MAVERRIC trial are expected in 2021 [55]. If MWA proves to be non-inferior to surgery, there will be higher level evidence that navigation guided MWA antenna placement is a good alternative to surgery.

Additional future perspective: circulating biomarkers

After reviewing the research on prognostication of patients after surgery for CRLM, one might speculate about the direction of future studies. Preferably, practitioners should be able to have accurate prognostic information shortly after patients are discharged from the hospital. In that case, decisions about adjuvant treatment and intensity of follow-up can be made without delay. After curative surgery for CRLM, patients are - macroscopically - free of tumour, but we know that more than 50% of patients will develop recurrent disease [56,57]. The question is in which organ or body fluid the micrometastases, which are too small to be detected by currently used CT/MR imaging, are hiding. Next, if we know where these tumour cells are hiding, are they able to form new metastases or will they eventually undergo apoptosis? Thus, is the detection of micrometastases sufficient for accurate prognostication or do these micrometastases also need to harbour certain molecular characteristics? In this thesis we analysed surgical tissue specimens for which multiple molecular markers were able to predict survival. However, biomarkers detected in blood, referred to as liquid biopsies, might be the future. Circulating biomarkers may yield information on tumour load and/or the presence of micrometastases that is not captured by surgical specimens.

Blood contains several components that can act as a source of biomarkers, e.g. circulating tumour cells (CTCs) [58], cell-free tumour DNA [59] and exosomes. Keratin 20 (CK20) is an epithelial protein used as a biomarker to detect CTCs. Multiple studies have reported the number of CTCs as an independent prognostic factor in unresectable metastatic CRC, which suggests that it might be a marker of tumour load [60–63]. After intentionally curative surgery, however, blood-derived CK20 did not qualify as a reliable prognostic biomarker [58,64–66]; it was, instead, bone-marrow-derived CK20 that was associated with a poor patient survival [64]. A second study also reported bone-marrow-detected cytokeratin as prognostic factor after surgery for CRLM [67]. Of note, the bone

marrow aspirations in these two studies were obtained just prior to surgery [64,67]. Unfortunately, bone marrow is not as easily accessible as the peripheral blood stream. However, if bone marrow is the site in which an accurate biomarker is detectable, it should be studied more thoroughly.

Cell-free DNA is mostly released by lymphocytes, but tumours also release their DNA by necrosis and apoptosis. The presence of circulating tumour DNA from CRC is predominantly identified using PCR-based methods in order to detect driver mutations in *KRAS* and *BRAF* [59,68,69]. Data on prognostication after surgery for CRLM by cell-free DNA is scarce. However, in those patients who had detectable circulating tumour DNA a few weeks after surgery for the primary CRC, 58%-94% developed recurrent disease. In contrast, only 0%-9.8% of the patients without circulating tumour DNA developed recurrences after surgery [69]. In a longitudinal follow-up after surgery or biopsy, tumour load can be monitored by quantifying the amount of tumour-specific mutations in a personalised PCR assay. This technique might therefore be suitable for prognostication and screening in the follow-up [59,68,69].

Exosomes are 40-100 nm vesicles produced by various types of cells, and they act like an envelope in which the content is hidden from the outside world [70]. Exosomes can contain DNA, mRNAs, microRNAs, proteins, lipids, carbohydrates, metabolites and viruses [70–72]. Tumours also secrete exosomes, which are detectable in the bloodstream [70,71]. The 'seed and soil' theory on the development of metastases described by Paget in 1889 suggests that the soil (liver parenchyma) is prepared for the seed (hematogenous dissemination of tumour cells) to arrive [73]. Current literature suggests that the content of exosomes might have a function in the preparation of the soil [48,74]. For example, tumours exposed to hypoxia produced exosomes with improved angiogenic and metastatic potential, which suggests that the tumour can adapt to a new situation and effectively nest at a new site [75,76]. Prognostication by exosomal content has not been studied in CRLM, but results in primary CRC look promising [77–79]. Future research is necessary to elucidate which exosomal content might lead to a successful preparation of the soil and the formation of new metastases. Circulating biomarkers show great promise and accurate predictions are hopefully within reach.

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